

Development of a Sterile Amniotic Membrane Tissue Graft Using Supercritical Carbon Dioxide

Jennifer L. Wehmeyer, PhD, Shanmugasundaram Natesan, PhD, and Robert J. Christy, PhD

Numerous techniques have been reported for preparing and sterilizing amniotic membrane (AM) for use in clinical applications. However, these preparations either do not produce completely sterile tissue or are detrimental to molecules unique to the tissue matrix, thus compromising beneficial wound-healing properties of the AM graft. The objective of this work was to produce a sterile human AM tissue graft using a novel preparation technique involving supercritical carbon dioxide (SCCO₂). AM tissue was subjected to various sterilization treatment groups that optimized the duration of exposure to SCCO₂ and the amount of peracetic acid (PAA) required to achieve a sterility assurance level of 10⁻⁶ log reduction in bacterial load. Effects of sterilization treatment on the histological, biophysical, and biochemical properties of the sterile AM were evaluated and compared with those of native AM tissue. Exposure of the AM tissue to combined SCCO₂ and PAA sterilization treatment did not significantly alter tissue architecture, the amounts of pertinent extracellular matrix proteins (type IV collagen, glycosaminoglycans, elastin) present in the tissue, or the biophysical properties of the tissue. AMs treated with SCCO₂ were also found to be excellent substrates for adipose-derived stem cell (ASC) attachment and proliferation *in vitro*. Human ASCs, attached to all treatment groups after 24 h of culture and continued to proliferate over the next few days. The current study's results indicate that SCCO₂ can be used to sterilize AM tissue grafts while simultaneously preserving their biological attributes. The preservation of these features make AM appealing for use in numerous clinical and tissue engineering applications.

Introduction

TEMPORARY DRESSINGS ARE important in the treatment of burn wounds because they help to reduce the risk of infection, prevent and minimize fluid loss, alleviate pain, and promote wound healing.^{1,2} Amniotic membrane (AM) has been used in numerous clinical wound-healing applications and has proven itself to be an efficacious biological dressing in the management of full- and partial-thickness burns, skin graft donor sites, and chronic leg ulcers.^{3,4} The therapeutic effects of AM are attributed to its biocompatibility; specifically, AM stimulates granulation tissue formation, promotes re-epithelialization of the wounded area, reduces scarring, and displays anti-inflammatory and anti-immunogenic properties.⁵ These benefits are due largely to the biological composition of AM as it contains several extracellular matrix (ECM) proteins, cytokines, growth factors, and other signaling molecules that are critical to cell infiltration, attachment, and proliferation that greatly impact the overall wound healing process.⁶⁻⁸

The preparation, preservation, and storage conditions of AMs are extremely important. Numerous techniques and methodologies have been reported, including treatment with

chemical detergents, gamma irradiation, and preservation in glycerol.⁹ Cryopreservation of tissue in glycerol is a commonly used technique (e.g., BioTissue, Inc., Miami, FL) because the ECM proteins and growth factors inherent to AM are maintained. However, cryopreservation with glycerol does not generate a sterilized product.¹⁰ Exposure to gamma irradiation is an effective method of sterilization, but results in severe degradation of the collagenous stroma of the AM.^{9,11} Therefore, careful selection of the processing and sterilization procedures for AM is of paramount importance for the preservation of key molecules unique to the tissue matrix.

An alternative to these traditional tissue processing techniques involves the use of supercritical carbon dioxide (SCCO₂). The term supercritical fluid denotes a substance that, at temperature and pressure conditions above its critical point, simultaneously exhibits gaseous viscosity and diffusion properties, but liquid densities and dissolution properties. Such characteristics can easily be manipulated with small changes in temperature and pressure, thus making supercritical fluids applicable to many industrial and laboratory processes.¹²⁻¹⁴

Of particular interest is the use of SCCO₂ in biomedical applications for processing both hard and soft human tissues

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 01 JUL 2015		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Development of a sterile amniotic membrane tissue graft using supercritical carbon dioxide				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Wehmeyer, J. L. Natesan, S. Christy, R. J.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) United States Army Institute of Surgica; Research, JBSA Fort Sam Houston, TX 78234				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 11	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

for transplantation. Specifically, SCCO₂ has been used for the successful delipidation of bone. Researchers found that upon exposure to SCCO₂ (with the addition of hydrogen peroxide), bone cells as well as the lipid fraction of the bone could be successfully removed.¹⁵ Others have used SCCO₂ to remove cell nuclei from soft tissue as well. In a study of porcine aortas exposed to SCCO₂, researchers were able to remove the nuclear material with 20 min of exposure.¹⁶

SCCO₂ can also be used to terminally sterilize medical devices, implants, and allograft tissues. After 1 h of exposure to SCCO₂, spore preparations of *Bacillus subtilis* and *Bacillus stearothermophilus* were inactivated and a sterility assurance level (SAL) of 10⁻⁶ (reduction in bacterial spore colony-forming units [CFUs]) was achieved. Scanning electron microscopy (SEM) revealed that the bacteria exposed to SCCO₂ remained intact; however, the lipid bilayer and internal cell structures were indistinguishable. Such results suggest that the mechanism of bacterial inactivation occurs through intracellular acidification due to enhanced mass transfer of CO₂ and disruption of the phospholipid bilayer.¹⁷

The use of SCCO₂ for preparing tissues for transplantation and other clinical applications is only beginning to be realized. SCCO₂ makes an attractive solvent for tissue processing for many reasons. First, SCCO₂ has low-viscosity and high-diffusion coefficients that allow it to penetrate solid microporous matrices, such as tissue ECM. In addition, CO₂ has relatively low critical coordinates (i.e., 93.2–98.7 atm and 35–39°C), making it ideal for delicate biological tissues. The low temperature of the process and the stability of CO₂ allow unwanted compounds, such as blood and lipids, to be extracted without compromising the physiological properties and mechanical integrity of the tissue.¹⁸ Furthermore, SCCO₂ is relatively nontoxic, thus allowing for maintained biocompatibility upon transplantation.

To date, the use of SCCO₂ to prepare and sterilize AM tissue for use in clinical applications has not been investigated, but is an appealing alternative to currently used AM tissue preparation methods. The objective of this study was to produce a sterile, biocompatible tissue allograft made of human AM that can be used in tissue engineering and regenerative medicine applications utilizing SCCO₂ technology.

Materials and Methods

Preparation of AM

Placentas from consenting mothers undergoing elective caesarean sections were acquired from the University Hospital (San Antonio, TX). The AM was isolated from the placenta using blunt dissection under sterile conditions. Once removed, the AM was thoroughly rinsed in saline to remove any remaining chorion, blood clots, and general debris. AM was placed epithelial side up on nitrocellulose paper (0.45 µm; Whatman), which were then sealed in DuPont™ Tyvek® packaging and kept frozen at –80°C before being exposed to SCCO₂.

Validation of tissue sterility

Tissue sterility experiments were carried out in accordance with industrial sterilization validation guidelines.^{19,20} Bacterial strains used included a clinical isolate of *Staphylococcus epidermidis* (obtained from San Antonio Military Medical Center, Fort Sam Houston, TX) and a spore suspension of *Clostridium sporogenes* (SGM Biotech/Mesa Labs, Bozeman, MT).

Each AM tissue piece, measuring 2.54 cm², was inoculated with 10⁶ CFU of either the *S. epidermidis* or *C. sporogenes*. Inoculated tissues were double packaged in Tyvek pouches and heat-sealed before exposure to the sterilization treatment. Sterilization consisted of exposing the tissue to SCCO₂ for 10–30 min with the addition of 0–2 mL of peracetic acid (PAA). Untreated AM tissue inoculated with bacteria/spores and the broth solutions alone served as controls. AM tissue was exposed to SCCO₂ using a Nova 2200 SCCO₂ sterilizer (NovaSterilis, Lansing, NY). The pressure and temperature of the SCCO₂ were held constant at 9900 kPa and 35°C, respectively (Fig. 1).

After sterilization, the AM tissues inoculated with *S. epidermidis* were placed in tubes containing nutrient broth and incubated at 37°C for 14 days. Similarly, tissue samples inoculated with *C. sporogenes* were placed in reinforced clostridium broth following sterilization and cultured under anaerobic conditions; that is, in an anaerobic chamber



FIG. 1. Schematic representation (not drawn to scale) of the Nova 2200 SCCO₂ sterilizer used to process amniotic membrane (AM) tissues. Color images available online at www.liebertpub.com/tec

(Whitley MG500; Don Whitley Scientific, Frederick, MD) under 10% hydrogen/10% CO₂/80% nitrogen for 14 days. The broth was monitored for bacterial growth by observing turbidity. For verification of growth, aliquots of broth from each sample were cultured on either nutrient or reinforced clostridial agar plates for *S. epidermidis* and *C. sporogenes*, respectively.

Histology

Native and SCCO₂-treated AM were fixed in 10% neutral buffered formalin overnight. The AM pieces were then embedded in paraffin by standard techniques and sectioned with a microtome. Cut sections were stained with picosirius red (PSR; Polysciences, Inc., Warrington, PA). PSR stain in conjunction with polarized light microscopy was used to enhance the inherent birefringence of collagen molecules, thus allowing for the evaluation of collagen fiber organization of SCCO₂-treated AM as compared with native tissue. Briefly, sections were deparaffinized and hydrated with distilled water and stained with Hematoxylin. Sections were then incubated in sirius red F3B solution for 1 h, washed in 0.01 N hydrochloric acid (HCl), and then dehydrated, cleared, and mounted. Images of the stained tissue sections were acquired with an Olympus BX60 microscope (Center Valley, PA) equipped with the appropriate filters for polarized light.

Scanning electron microscopy

Native and SCCO₂-treated AM tissues were prepared for SEM following standard procedures.²¹ Briefly, tissues were fixed in 2.5% phosphate buffered glutaraldehyde at 4°C for 1 h, dehydrated in a series of graded alcohols (50%, 70%, 80%, 90%, 95%, and 100%) and dried using hexamethyldisilazane (Electron Microscopy Sciences, Hatfield, PA). The samples were then sputter coated with a thin layer (10 nm) of gold and palladium (Anatech, Union City, CA), and examined on a Zeiss Sigma VP 40 (Zeiss-Leica, Thornwood, NY) scanning electron microscope.

Immunohistochemical staining

The ECM of SCCO₂-treated AM tissue was also evaluated immunohistochemically. Briefly, cleared and dehydrated AM tissue sections were rinsed in a solution of Tris buffered saline (TBS; Fisher, Fair Lawn, NJ) with 0.025% Triton X-100 (Sigma Aldrich, St. Louis, MO) and then blocked for 2 h at room temperature using a solution of 10% horse serum (Gibco, Grand Island, NY) and 1% bovine serum albumin (BSA; Sigma Aldrich) in TBS. Following blocking, tissue sections were incubated with the primary antibody, a mouse monoclonal antibody specific to type I collagen (Abcam, Cambridge, MA), diluted 1:400 in TBS with 1% BSA at 4°C overnight. Next, sections were rinsed in TBS/0.025% Triton X-100 and then incubated in 0.3% hydrogen peroxide (Henry Schein, Melville, NY) at room temperature for 15 min to block endogenous peroxidase activity. Sections were washed in TBS and incubated with a biotinylated horse anti-mouse IgG secondary antibody (1:250 dilution; Vector, Burlingame, CA) at room temperature for 1 h, rinsed in TBS again, then incubated in Vecstatin ABC reagent (Vector) at 37°C for 30 min before development with diaminobenzidine (Vector).

The sections were finally rinsed in running tap water and counterstained with methyl green (Vector). Stained sections were then dehydrated, cleared, and mounted. Images of the stained tissue sections were acquired with an Olympus BX60 microscope.

The plasma membranes of the epithelium of native and SCCO₂-treated AMs were fluorescently labeled. Briefly, whole pieces of AM tissue were fixed in acetone at -20°C for 3 min, rinsed in Hank's balance salt solution (HBSS; Life Technologies, Eugene, OR) and then incubated at 37°C in 10 µg/mL of CellMask™ Deep Red Plasma membrane stain (Molecular Probes) solution for 5 min. The labeled AM tissues were again rinsed in HBSS before being mounted in ProLong Gold Antifade reagent (Molecular Probes) and imaged. Fluorescent images of the cell membranes were obtained using the appropriate filters (649/666 nm excitation/emission).

Differential scanning calorimetry

The thermal transitions of the SCCO₂-treated AMs were analyzed by differential scanning calorimetry (DSC) using a Perkin Elmer DSC7 (Waltham, MA). Native and SCCO₂-treated AM tissues were lyophilized overnight, weighed (5 mg dry weight per sample), then sealed in aluminum pans before being heated at a rate of 30°C/min over a temperature range of 25–300°C. An empty aluminum pan served as the reference for all samples tested. DSC thermograms were collected using the accompanying Pyris software, and the temperatures at which the thermal transition peaks occurred were identified. The transition temperatures, an indicator of the resistance of a material to heat denaturation, were defined as the peak maximum of the resultant endothermic peaks. Results from three separate trials were averaged. A second thermal run on the native tissue was performed to compare the transitions of a denatured tissue sample.

Fourier Transform Infrared spectroscopy

Changes in the chemical structures of AM after treatment with SCCO₂ were evaluated using the Fourier Transform Infrared (FTIR) spectroscopy. Spectra for native and SCCO₂-treated AM were acquired using a Tensor 27 spectrometer (Bruker, Billerica, MA). Spectral scanning in the range of 4500–400 cm⁻¹ with a resolution of 4 cm⁻¹ was performed and the absorbance at each wavelength recorded for all samples using the OPUS software.

Biochemical characterization

The amounts of hydroxyproline, type IV collagen, elastin, and glycosaminoglycans (GAGs) present in native (*n* = 6) and SCCO₂-treated tissues (*n* = 6) were quantified with commercially available assays: hydroxyproline (BioVision, Mountain View, CA); type IV collagen enzyme-linked immunosorbent assay (ELISA) (Exocell, Philadelphia, PA); Fastin™ Elastin Assay (Biocolor, Northern Ireland, United Kingdom); and Blyscan sulfated GAG assay (Biocolor), respectively. All assays were conducted following the manufacturer's recommended procedures. Briefly, tissue samples were lyophilized overnight, and the dry weight measured. For the hydroxyproline assay, the AM tissue was completely solubilized in 12 N HCl at 100°C for 3 h. For the extraction of type IV collagen and the GAGs, the

tissue was digested in a papain extraction reagent consisting of 0.2 M sodium phosphate buffer, sodium acetate, ethylenediaminetetraacetic acid (EDTA), cysteine HCl, and papain at 65°C overnight. Elastin was extracted by incubating tissue samples in 0.25 M oxalic acid at 60°C for 1 h. The elastin extraction process was repeated with fresh oxalic acid, and the two extractions were pooled for analysis. The concentrations of hydroxyproline, type IV collagen, elastin, and GAGs contained in each sample tested were determined using a standard curve of light absorbance (560, 450, 513, and 656 nm for hydroxyproline, type IV collagen, elastin, and GAGs, respectively) versus known concentrations of each protein run in parallel with the experimental samples.

The degree of collagen denaturation after SCCO₂ treatment was also assessed using an α -chymotrypsin assay following previously published procedures.²² Briefly, lyophilized AM was incubated in 0.1 M tris HCl containing 1 mg/mL α -chymotrypsin (Sigma Aldrich), 1 mM iodoacetamide, and 1 mM ethylenediaminetetraacetic acid (Sigma Aldrich) overnight at 37°C to digest denatured collagen within the matrix. The supernatant, containing the degraded collagen, was solubilized and the amount of hydroxyproline determined. The amount of hydroxyproline obtained from denatured collagen was expressed as a percentage of the total hydroxyproline content.

Adipose-derived stem cell culture

The biocompatibility of AM tissue exposed to SCCO₂ was evaluated *in vitro* using human adipose-derived stem cells (hASCs) previously isolated in our laboratory.²³ The hASCs were maintained in MesenPRO RS™ basal medium supplemented with MesenPRO RS growth supplement, an antibiotic-antimycotic solution (100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B), and 2-mM L-glutamine (Gibco, Invitrogen, Carlsbad, CA) under standard cell culture conditions (i.e., a sterile, 37°C, humidified, 5% CO₂/95% air environment). Cells at passage 2–4 were used in experiments.

ASC attachment and proliferation

SCCO₂-treated AMs were placed inside 12-well cell culture inserts (BD Biosciences, Franklin Lakes, NJ) with the epithelial side facing upward. The hASCs fluorescently labeled with carboxyfluorescein diacetate, succinimidyl ester (Life Technologies) were seeded (50,000 cells per insert) on top of the AMs and maintained, submerged in culture medium, over a period of 4 days. Cell attachment was assessed 24 h postseeding, and fluorescent images were taken with an Olympus IX71 inverted microscope (Olympus America, Inc., Center Valley, PA).

Proliferation was monitored over the 4-day period using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] metabolic activity assay (Life Technologies). Briefly, on days 1, 2, 3, and 4, MTT (5 mg/mL) was added to the inserts. The hASCs were allowed to reduce the tetrazolium salt to formazan over a period of 4 h. At that time, the purple formazan was extracted from the cells and solubilized using dimethyl sulfide (Sigma, St. Louis, MO). The optical density of the resulting solution was determined by measuring the absorbance at 570 nm

(630 nm reference) using a microplate reader (Synergy MX; BioTek, Winooski, VT).

Statistical analyses

Statistical analyses of numerical results were performed using the GraphPad Prism (GraphPad Software, Inc., San Diego, CA) statistical software package. Numerical data are expressed as mean \pm standard error of the mean. Comparisons between groups were made using a one-way analysis of variance (ANOVA) with values of $p \leq 0.05$ considered statistically significant. *Post hoc* analyses were performed using the Tukey's multiple comparison test.

Results

Tissue sterility

Sterilization of AM inoculated with *S. epidermidis* and *C. sporogenes* was achieved with minimal processing time and minimal amounts of PAA sterilizing agent (Table 1). SCCO₂ alone without the addition of the sterilizing agent PAA was not sufficient to inactivate the *S. epidermidis* and *C. sporogenes* bacteria as evidenced by bacterial growth detected 3 days poststerilization treatment. A volume of 0.5 mL of PAA with 10 min of exposure to SCCO₂ was sufficient to inactivate *S. epidermidis* and inhibit contamination over the 2-week culture period; however, an increase in the duration of SCCO₂ treatment and volume of PAA additive was needed to inactivate *C. sporogenes*. The amount of PAA needed to inactivate both *S. epidermidis* and *C. sporogenes* in the shortest time possible (10 min of SCCO₂ exposure) required using 2 mL of PAA. These treatment parameters were deemed optimal for the production of sterile AM tissue. AMs exposed to this sterilization treatment were further characterized.

TABLE 1. EFFECTIVENESS OF SCCO₂ AND PAA ON BACTERIAL INACTIVATION

Amount of PAA (mL)	Duration of exposure to SCCO ₂ (min)	Staphylococcus epidermidis	Clostridium sporogenes
0	10	Fail	Fail
0	20	Fail	Fail
0	30	Fail	Fail
0.5	10	Pass	Fail
0.5	20	Pass	Fail
0.5	30	Pass	Fail
1	10	Pass	Fail
1	20	Pass	Pass
1	30	Pass	Pass
2	10	Pass	Pass
2	20	Pass	Pass
2	30	Pass	Pass

Two processing parameters, peracetic acid (PAA) and duration of exposure to supercritical carbon dioxide (SCCO₂), were evaluated to determine their effectiveness of eliminating bacterial survivors from inoculated AM tissue. "Pass" denotes the absence of bacterial growth on the agar plates after the prescribed culture time, whereas "Fail" represents observed bacterial growth on the agar culture plates indicating one or more bacterial survivors.

Histological evaluation of the ECM of SCCO₂-treated AM

Histological comparisons between native and SCCO₂-treated AM tissue samples were performed to determine the effects of SCCO₂ treatment on the gross appearance and general structural properties of the tissue ECM (Fig. 2). Sterilization did not affect the tissue structure and collagen organization (Fig. 2A, D). PSR staining of SCCO₂-treated tissue under polarized light revealed dispersed birefringence with green, yellow, and orange/red coloring present throughout most of the tissue regions (Fig. 2B, E). The color and intensity of the birefringence were similar to that of native AM. Immunohistochemical observation of SCCO₂-treated AM showed positive staining for the major ECM protein collagen I throughout the entire cross section of tissue, similar to that of native AM (Fig. 2E, F).

Assessment of the SCCO₂-treated amniotic epithelium

The structure and ultrastructure of the AM epithelium were evaluated pre- and poststerilization treatment with SEM and fluorescent microscopy. The epithelium after SCCO₂ treatment appeared flattened with less distinguishable cellular boundaries as compared with native epithelium (Fig. 3A, B). Further examination of the plasma membrane

revealed the SCCO₂-treated group had no positive staining for membrane lipids (Fig. 3C, D).

DSC and FTIR spectroscopy analyses of native and SCCO₂-treated AM

DSC was used to analyze the thermal transitions of native and SCCO₂-treated AM tissues. As the AM tissue was subjected to a constant heating rate (30°C/min), the collagen molecules present in the tissue underwent thermal dehydration and structural changes as evidenced by the endothermic peaks that appeared on the DSC curves (Fig. 4A). The first thermal transition occurred at 114.4°C±0.68°C and 113.4°C±0.88°C for native and SCCO₂-treated membranes, respectively. The second thermal transition occurred at 218.4°C±0.92°C and 220.7°C±1.783°C for native and SCCO₂-treated membranes, respectively. DSC of denatured native AM did not show characteristic transition peaks. SCCO₂-treated membranes exhibited amide absorption bands at 1654 cm⁻¹ (amide I), 1552 cm⁻¹ (amide II), and 1238 cm⁻¹ (amide III) similar to those observed in the native membrane (Fig. 5). Other peaks present in the spectra of both native and SCCO₂-treated AMs include 3315 and 2925 cm⁻¹, corresponding to N-H (amine) and C-H (alkane) stretching vibrations (which are associated with lipid alkyl chains), respectively. There were no qualitative

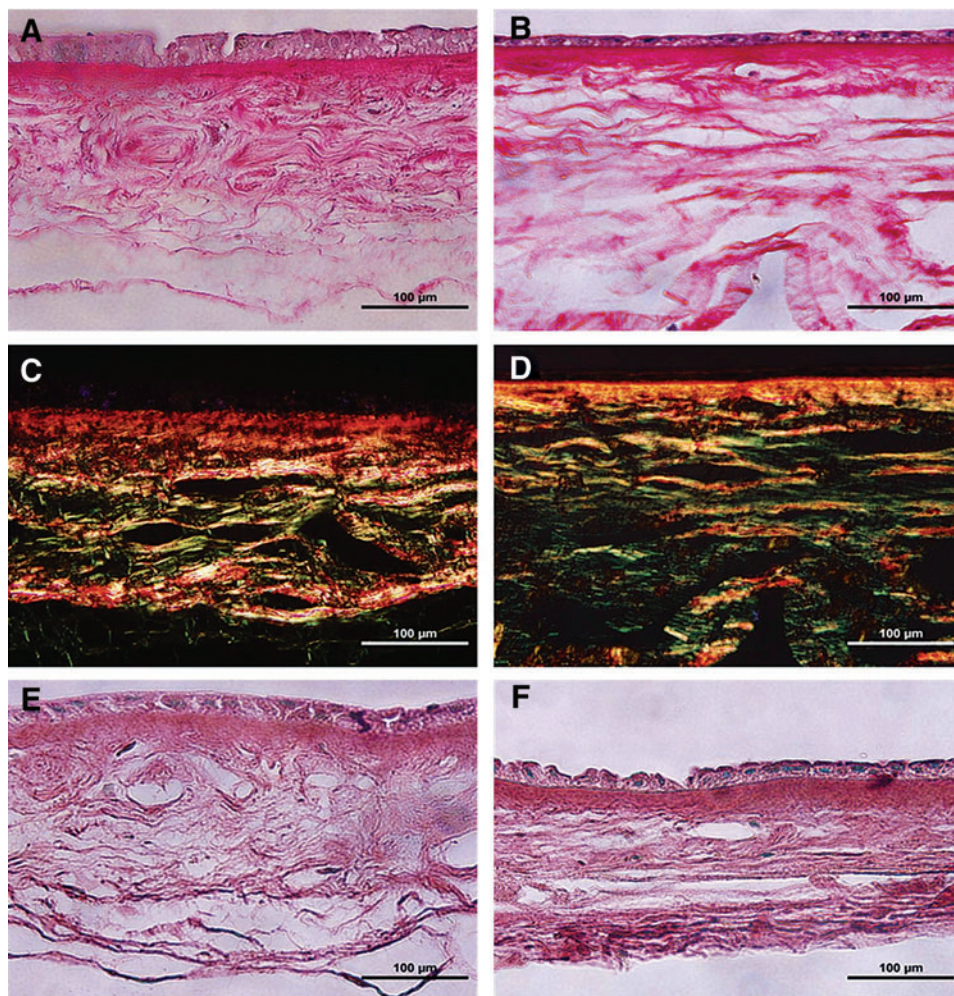
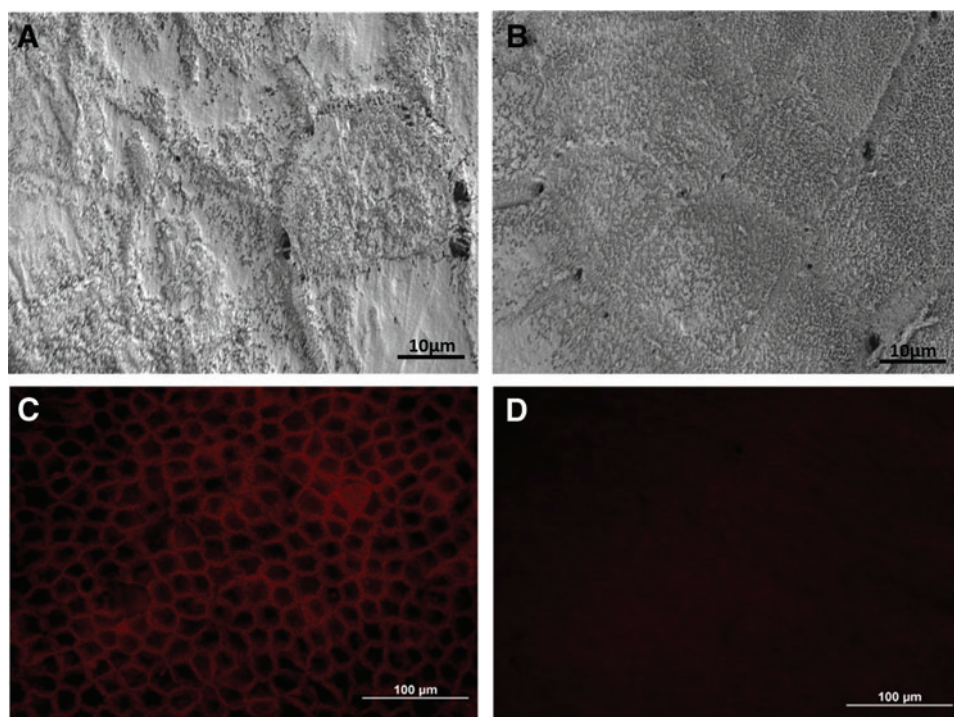


FIG. 2. Light (A, B, E, F) and polarized light (C, D) micrographs of native (left column) and SCCO₂-treated (right column) AM. Picrosirius red-stained supercritical carbon dioxide (SCCO₂)-treated AM (B) showed similar gross structural appearance as compared with native (A). When viewed under polarized light, a similar pattern of birefringence is observed for both native (C) and SCCO₂-treated (D) AM. Light micrographs of native (E) and SCCO₂-treated (F) AM revealed positive immunohistochemical staining for collagen I. Color images available online at www.liebertpub.com/tec

FIG. 3. Scanning electron micrograph images of native (A) and SCCO₂-treated (B) AM revealed changes in the ultrastructure of amniotic epithelium with the SCCO₂-treated group, the cells have a flattened appearance. The epithelium was fluorescently labeled using the CellMask™ Deep Red Plasma membrane stain (C, D) which showed the plasma membrane was not intact post SCCO₂ sterilization treatment (D). Color images available online at www.liebertpub.com/tec



differences in the spectral peak positions or the pattern of peaks present for the native and SCCO₂-treated tissue.

To verify the findings of DSC and FTIR and ensure that the denaturation of collagen proteins was not occurring as a result of the SCCO₂ treatment, the amount of denatured collagen was estimated using an α -chymotrypsin/hydroxyproline assay. The amounts of hydroxyproline from denatured collagen in native AM tissue and from AM tissue that underwent SCCO₂ sterilization were not significantly different. Native AM consisted of 4% hydroxyproline from denatured collagen while SCCO₂-treated AM contained 5.1% hydroxyproline from denatured collagen (Fig. 4B).

Biochemical analyses of SCCO₂-treated AM

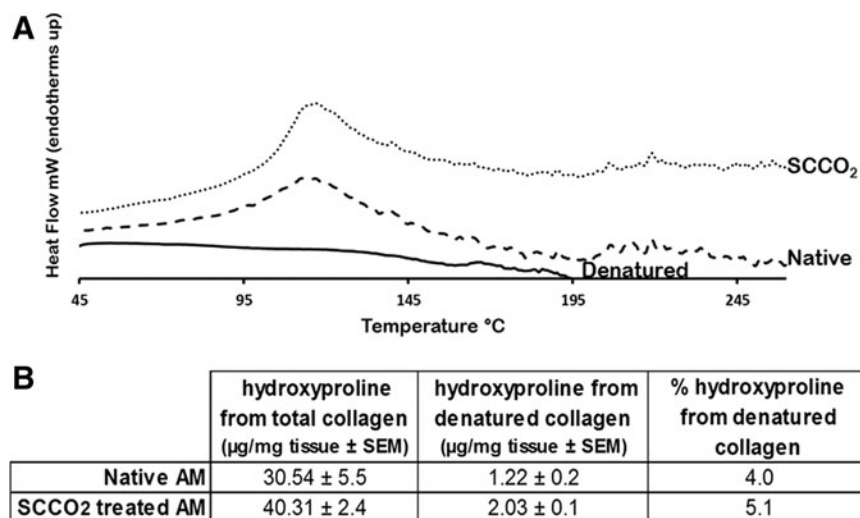
Multiple measures of ECM components (hydroxyproline, type IV collagen, elastin, and GAGs) did not significantly

differ ($n=6$; $p>0.05$) between native and SCCO₂-treated AM (Fig. 6). Hydroxyproline present in native and SCCO₂-treated AM was 30.54 ± 5.48 and 40.31 ± 2.36 $\mu\text{g}/\text{mg}$ of tissue, respectively. Type IV collagen present in native and SCCO₂-treated AM was 4.0 ± 0.7 and 4.5 ± 0.8 ng/mg of tissue, respectively. The amount of sulfated GAGs present in native and SCCO₂-treated AM was 10.91 ± 0.77 and 13.41 ± 1.91 $\mu\text{g}/\text{mg}$ of tissue, respectively. Elastin in native and SCCO₂-treated AM was 147.0 ± 21.0 and 109.6 ± 20.7 $\mu\text{g}/\text{mg}$ of tissue, respectively.

In vitro biocompatibility of SCCO₂-treated AM

After 24 h of culture hASCs attached to SCCO₂-treated AM (Fig. 7A), and continued to proliferate over the next few days (Fig. 7B) as determined by MTT. By day 4, the hASCs had more than doubled in population.

FIG. 4. Differential scanning calorimetry was used to obtain representative thermograms of denatured, native, and SCCO₂-treated AM (A). An α -chymotrypsin/hydroxyproline assay was used to estimate the amount of denatured collagen in SCCO₂-treated AM tissue (B). Numerical data are expressed as mean \pm standard error of the mean of six different samples. Comparisons between groups were made using a t-test with values of $p \leq 0.05$ considered statistically significant.



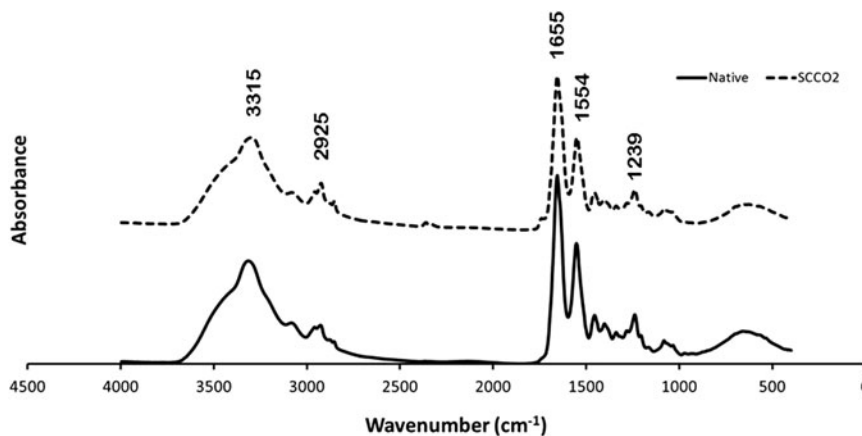


FIG. 5. Infrared spectra of native AM and AM subjected to SCCO₂ exposure. Peaks in the spectra, corresponding to major function groups present in the tissue, are similar for both native and SCCO₂-treated AM suggesting that the functional groups present in the tissue are not affected by sterilization treatment.

Discussion

The detrimental effects that occur as a result of sterilization treatment have the potential to alter or impair the desired function of AM tissue postimplantation. In an attempt to overcome these limitations, we utilized SCCO₂ for the sterilization of AM and then evaluated the effects of SCCO₂ treatment on the physical, chemical, and biological properties of the tissue. The results of this study demonstrate SCCO₂, when used with PAA, is an effective sterilization method that meets industrial sterility standards (i.e., SAL of 10⁻⁶). Furthermore, this method of sterilization is not damaging to the structural and biological properties of the tissue, thus making SCCO₂-treated AM appealing for clinical applications.

AM tissues have found utility as tissue engineering substrates for regenerative purposes because of their unique ECM compositions.^{24,25} As such, the methodologies used for the preparation, preservation, and storage of AM are extremely important. Cryopreservation in glycerol is the most widely used method of AM tissue graft preservation, introduced by Tseng and colleagues in 1995.²⁶ Since then, numerous studies

have shown that cryopreserved AM is similar to native AM with regard to its structural architecture and biological composition.^{7,9,27,28} In addition, cryopreserved AM has demonstrated clinical success when used therapeutically for corneal epithelial defects, cutaneous partial thickness burns, and conjunctival surface reconstruction.²⁹⁻³¹ However, there exists a fine balance between maintaining the biological and structural integrity of the tissue matrix and meeting the regulatory requirements for sterility imposed upon by the FDA and the American Association of Tissue Banks for the prevention of disease transmission. Although cryopreservation of AM in glycerol is advantageous from a tissue/material perspective, this method, does not ensure complete tissue sterility.^{11,32,33}

Unlike metals and plastics, the sterilization of soft, biological tissues have many challenges. First, the biomechanical and biological properties of the tissue can be adversely affected. For example, gamma irradiation and steam and heat sterilization of soft tissues, and in particular AM, cause significant structural damage through irreversible degradation of ECM proteins.^{9,34} Furthermore, traditional sterilization methods do not penetrate the tissue adequately

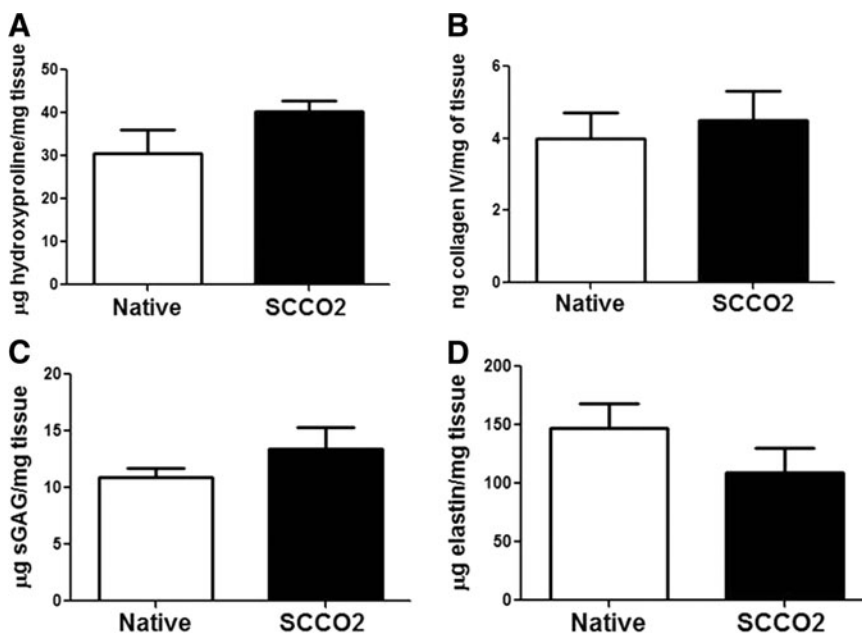
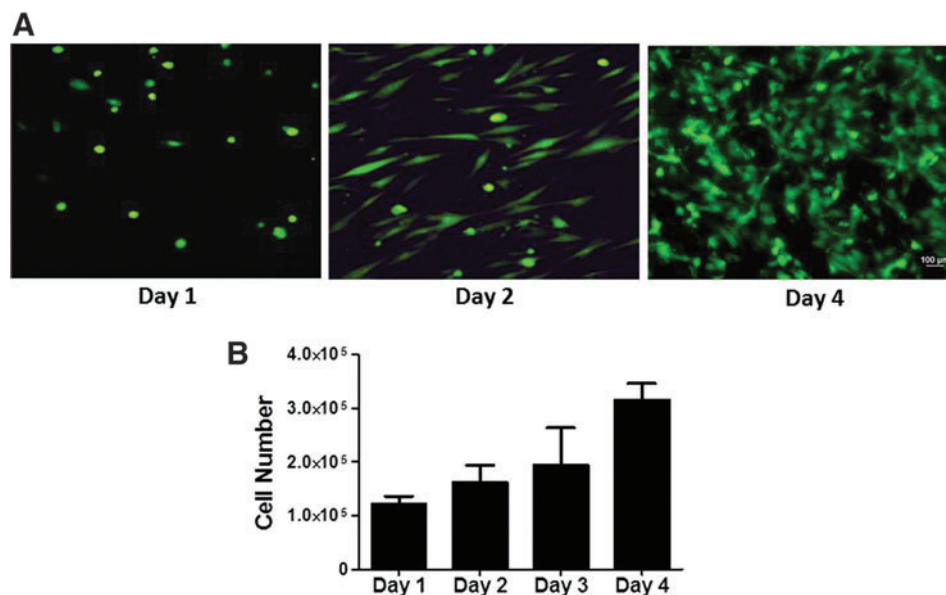


FIG. 6. Hydroxyproline (A), collagen IV (B), sulfated glycosaminoglycans (sGAG) (C), and elastin (D) content of native and SCCO₂-treated AM tissues were determined by using colorimetric methods and ELISA (collagen IV only). There were no significant differences between the native and sterilized AM. The data represents mean values (\pm standard error of the mean) of six different AM samples. All of the values are expressed per milligram of tissue.

FIG. 7. Representative fluorescent micrograph of human adipose-derived stem cells (hASCs) seeded on SCCO₂-treated AM tissue over a period of 4 days of culture (A). The cells were labeled with carboxyfluorescein succinimidyl ester before seeding and were seeded at a density of 50,000 cells per sample. The hASCs proliferation was monitored with the MTT assay (B). Uptake of the dye increased over a period of 4 days, thus indicating increased cell proliferation. Each bar represents the mean \pm standard error of the mean of three observations. Color images available online at www.liebertpub.com/tec



enough to kill bioburden residing deep within the tissue matrix. Tissue complexity pertinent to intricate ECM architectures along with the presence of an array of organic biomolecules and cells, aids in protecting the bioburden thus making sterilization more difficult.³⁵ For these reasons, there exists a need for more advanced sterilization technologies that can be used in conjunction with human tissue grafts, more specifically, delicate human AM tissue. Our results showed that sterilization of AM with SCCO₂ overcomes the limitations associated with other sterilization methods specifically in that the SCCO₂ process maintained the structural integrity of the tissue matrix and preserved key ECM proteins while simultaneously inactivating the bioburden. Like other processes currently used in the healthcare industry for the sterilization of tissues and medical devices, SCCO₂ sterilization must be validated in accordance with industrial sterilization standards.^{19,20} Specifically, a SAL of 10^{-6} (defined as the probability of a contaminant existing after sterilization treatment to be one in a million after a 10^6 log reduction in bacterial load)¹⁷ must be achieved in order for a claim of sterility to be made. In addition, the bacterial species used in such sterilization validations must be a species that exhibits extreme resistance to inactivation. Spore-forming bacteria are commonly used for this purpose because of their resistance to traditional sterilization processes.^{36,37} In the current study, we used SCCO₂ with minimal amounts of PAA to achieve a SAL 10^{-6} against both *S. epidermidis* (a prevalent bacterial species found on AM) and *C. sporogenes* (spore-forming bacteria resistant to antimicrobial/sterilization treatments) to meet industrial sterilization requirements. Although four times as much PAA was required for inactivation of *C. sporogenes*, the amount of PAA used in this study is still significantly less than the amounts typically reported (0.1%) when PAA alone is used for sterilization.²⁴ Furthermore, combining PAA with SCCO₂ resulted in a relatively short exposure time of the tissue to PAA. By comparison, using PAA alone for the sterilization of tissues, such as skin grafts or urinary bladder matrix, requires anywhere from 3 to 5 h of processing for sterilization to be achieved.^{9,24,38,39}

To ensure that the sterilization treatment did not result in AM degradation or loss of ECM proteins, we performed microscopic, spectroscopic, thermodynamic, and biochemical analyses on native and SCCO₂-treated AMs. IR spectroscopy is a useful tool for obtaining molecular-level information pertinent to functional groups, chemical bonds, and molecular confirmations of proteins present in normal, physiological, and pathological tissues.^{40,41} Both FTIR and DSC analyses were carried out to show that SCCO₂ treatment imposes minimal to no changes in molecular organization of ECM protein present in AM. Specifically, FTIR was used as an analytical measure to confirm the presence of collagen, the predominant ECM protein of AM, through signature peaks that are unique to the protein. FTIR spectra of AM tissue treated with SCCO₂ and PAA did not show any considerable differences in the absorption frequencies and peak intensities in comparison to native tissues. Moreover, FTIR analysis did not show any major change in the spectral frequencies corresponding to amide absorption peaks typical to AM and other collagenous tissues.⁴² Complementing the FTIR findings, observations of the DSC thermograms confirmed that SCCO₂ treatment does not affect essential ECM elements present within AM. These findings were further validated through DSC analyses and quantified using a collagen degradation assay. Thermal denaturation involves the breaking of intramolecular bonds of the collagen molecules present in the AM tissue, reducing the organized triple helices to a random, amorphous coil form. Any degradation to the collagen molecule brought about as a result of processing techniques would result in a decrease in the thermal transition temperatures of the AMs; however, because no significant changes were observed in the DSC curves between the native and the SCCO₂-treated AM tissues, we can conclude that the sterilization treatment is not adversely affecting the hydrothermal stability of the AM tissue.

Native amnion consists primarily of type I and III collagens with smaller amounts of collagen types IV–VII. In addition, AM contains significant amounts of the proteoglycans

decorin, biglycan, and hyaluronic acid, as well as GAGs and elastin molecules.^{43–45} Collectively, these biomolecules not only provide tensile strength and impart elasticity to the AM, but facilitate cell function as well.^{6,46} Higa *et al.* showed greater lymphocyte adhesion to areas of AM ECM containing high levels of the GAG hyaluronic acid, suggesting the entrapment of inflammatory cells to the AM stroma may have a role in suppressing inflammation and fibrosis.⁴⁷ While the exact mechanisms are not clearly understood, it is suggested that the presence of antiangiogenic proteins and various growth factors in AM contribute to the beneficial therapeutic effects observed when AM is used clinically in cutaneous wound healing and ocular applications.^{7,8,28} Although further biochemical testing should be performed to confirm select growth factors and cytokines are present in SCCO₂-treated AM, our current findings suggest the SCCO₂ sterilization methodology is compatible with the delicate AM tissue with regard to preserving key biochemical components of the matrix.

Since SCCO₂ sterilization treatment did not cause major structural and biological degradation, the treated membranes were well suited to promote desirable cell interactions. We showed that SCCO₂-treated AM acts as a bioactive substrate for stem cell attachment and proliferation, making the tissue appealing for wound healing and regenerative medicine applications. It is well known that the degree of cellular attachment, migration, and proliferation on a biological scaffold is mediated by the scaffold's ECM milieu, and in addition, the conditions used to process AM significantly affect cell function *in vitro*. A recent report showed that limbal epithelial stem cells cultured on glycerol-preserved membrane tissues were less confluent, exhibited lower cell density, and expressed less of their characteristic stem cell markers than cells cultured on AM cryopreserved in buffer alone.³² In the present study, we showed that hASCs readily attached to the sterilized AM after 24 h, exhibited typical fibroblast-like morphology, and continued to proliferate over time. This shows the potential of AM to act not only as a bioactive cover, but also as a delivery vehicle by which the patient's own cells can be transplanted to the injured area. The SCCO₂-treated AM is a biomaterial well suited to form the basis of a skin equivalent and may act as a viable substrate for the treatment of a variety of conditions, including burns, ulcers, and for normal physiological wound healing.

Conclusions

The use of SCCO₂ and PAA presents a novel single-step technique to process AM tissue for potential clinical application. SCCO₂ treatment allows for AM to be processed such that the tissue graft is not only sterile, but also has the physical and biochemical properties that are most similar to that of native tissue. Furthermore, SCCO₂ acts as an excellent scaffold for stem cell attachment and proliferation, which potentially could be used as a tissue-engineered substitute for use in wound healing and regenerative medicine applications.

Acknowledgments

The authors would like to thank the Alamo Tissue Service for use of their equipment and facilities; Dr. Tao You of the USAISR for assistance acquiring the SEM images; Ms.

Sandra Becerra of the USAISR for her technical support; and Dr. Henry Rawls of the University of Texas Health Science Center San Antonio for use of his DSC equipment. Jennifer Wehmeyer's funding was provided by the Clinical and Rehabilitative Medicine Research Program, U.S. Army Medical Research and Materiel Command.

Disclaimer

The opinions and assertions contained in this study are the private views of the authors and are not to be construed as official or reflecting the views of the Department of Defense or Department of Army. The authors are employees of the U.S. Government, and this work was prepared as part of their official duties. This research was funded by the U.S. Army Medical Research and Materiel Command. This study was conducted under a protocol reviewed and approved by the U.S. Army Medical Research and Materiel Command Institutional Review Board and in accordance with the approved protocol.

Disclosure Statement

No competing financial interests exist.

References

1. Jones, V., Grey, J.E., and Harding, K.G. ABC of wound healing—wound dressings. *Br Med J* **332**, 777, 2006.
2. Lineen, E., and Namias, N. Biologic dressing in burns. *J Craniofac Surg* **19**, 923, 2008.
3. Branski, L.K., Herndon, D.N., Celis, M.M., Norbury, W.B., Masters, O.E., and Jeschke, M.G. Amnion in the treatment of pediatric partial-thickness facial burns. *Burns* **34**, 393, 2008.
4. Mermet, I., Pottier, N., Sainthillier, J.M., Malugani, C., Cairey-Remonnay, S., Maddens, S., Riethmuller, D., Tiberghien, P., Humbert, P., and Aubin, F. Use of amniotic membrane transplantation in the treatment of venous leg ulcers. *Wound Repair Regen* **15**, 459, 2007.
5. Niknejad, H., Peirovi, H., Jorjani, M., Ahmadiani, A., Ghanavi, J., and Seifalian, A.M. Properties of the amniotic membrane for potential use in tissue engineering. *Eur Cell Mater* **15**, 88, 2008.
6. Bryant-Greenwood, G.D. The extracellular matrix of the human fetal membranes: structure and function. *Placenta* **19**, 1, 1998.
7. Koizumi, N.J., Inatomi, T.J., Sotozono, C.J., Fullwood, N.J., Quantock, A.J., and Kinoshita, S. Growth factor mRNA and protein in preserved human amniotic membrane. *Curr Eye Res* **20**, 173, 2000.
8. Hopkinson, A., McIntosh, R.S., Shanmuganathan, V., Tighe, P.J., and Dua, H.S. Proteomic analysis of amniotic membrane prepared for human transplantation: characterization of proteins and clinical implications. *J Proteome Res* **5**, 2226, 2006.
9. von Versen-Hoynck, F., Syring, C., Bachmann, S., and Moller, D.E. The influence of different preservation and sterilisation steps on the histological properties of amnion allografts—light and scanning electron microscopic studies. *Cell Tissue Bank* **5**, 45, 2004.
10. van Baare, J., Ligtvoet, E.E., and Middelkoop, E. Microbiological evaluation of glycerolized cadaveric donor skin. *Transplantation* **65**, 966, 1998.
11. Riau, A.K., Beuerman, R.W., Lim, L.S., and Mehta, J.S. Preservation, sterilization and de-epithelialization of human

- amniotic membrane for use in ocular surface reconstruction. *Biomaterials* **31**, 216, 2010.
12. Reverchon, E., and De Marco, I. Supercritical fluid extraction and fractionation of natural matter. *J Supercrit Fluid* **38**, 146, 2006.
 13. Reverchon, E., Adami, R., Cardea, S., and Della Porta, G. Supercritical fluids processing of polymers for pharmaceutical and medical applications. *J Supercrit Fluid* **47**, 484, 2009.
 14. Marr, R., and Gamse, T. Use of supercritical fluids for different processes including new developments—a review. *Chem Eng Process* **39**, 19, 2000.
 15. Fages, J., Marty, A., Delga, C., Condoret, J.S., Combes, D., and Frayssinet, P. Use of supercritical CO₂ for bone delipidation. *Biomaterials* **15**, 650, 1994.
 16. Sawada, K., Terada, D., Yamaoka, T., Kitamura, S., and Fujisato, T. Cell removal with supercritical carbon dioxide for acellular artificial tissue. *J Chem Technol Biotechnol* **83**, 943, 2008.
 17. White, A., Burns, D., and Christensen, T.W. Effective terminal sterilization using supercritical carbon dioxide. *J Biotechnol* **123**, 504, 2006.
 18. Nichols, A., Burns, D., and Christopher, R. Studies on the sterilization of human bone and tendon musculoskeletal allograft tissue using supercritical CO₂. *J Orthop* **6**, 12, 2009.
 19. ISO 11137-2: 2006: Sterilization of health care products—radiation. Part 2: establishing the radiation dose.
 20. ISO 14937: 2000: Sterilization of health care products. General requirements for characterization of a sterilizing agent and the development, validation and routine control of a sterilization process for medical devices.
 21. Araujo, J.C., Teran, F.C., Oliveira, R.A., Nour, E.A., Montenegro, M.A., Campos, J.R., and Vazoller, R.F. Comparison of hexamethyldisilazane and critical point drying treatments for SEM analysis of anaerobic biofilms and granular sludge. *J Electron Microsc* (Tokyo) **52**, 429, 2003.
 22. Bank, R.A., Krikken, M., Beekman, B., Stoop, R., Maroudas, A., Lafeber, F.P., and te Koppele, J.M. A simplified measurement of degraded collagen in tissues: application in healthy, fibrillated and osteoarthritic cartilage. *Matrix Biol* **16**, 233, 1997.
 23. Natesan, S., Baer, D.G., Walters, T.J., Babu, M., and Christy, R.J. Adipose-derived stem cell delivery into collagen gels using chitosan microspheres. *Tissue Eng Part A* **16**, 1369, 2010.
 24. Wilshaw, S.P., Kearney, J., Fisher, J., and Ingham, E. Biocompatibility and potential of acellular human amniotic membrane to support the attachment and proliferation of allogeneic cells. *Tissue Eng Part A* **14**, 463, 2008.
 25. Hopkinson, A., Shanmuganathan, V.A., Gray, T., Yeung, A.M., Lowe, J., James, D.K., and Dua, H.S. Optimization of amniotic membrane (AM) denuding for tissue engineering. *Tissue Eng Part C* **14**, 371, 2008.
 26. Kim, J.C., and Tseng, S.C.G. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. *Cornea* **14**, 473, 1995.
 27. Thomasen, H., Pauklin, M., Noelle, B., Geerling, G., Vetter, J., Steven, P., Steuhl, K.P., and Meller, D. The effect of long-term storage on the biological and histological properties of cryopreserved amniotic membrane. *Curr Eye Res* **36**, 247, 2011.
 28. Hao, Y.X., Ma, D.H.K., Hwang, D.G., Kim, W.S., and Zhang, F. Identification of antiangiogenic and anti-inflammatory proteins in human amniotic membrane. *Cornea* **19**, 348, 2000.
 29. Tseng, S.C.G., Prabhasawat, P., and Lee, S.-H. Amniotic membrane transplantation for conjunctival surface reconstruction. *Am J Ophthalmol* **124**, 765, 1997.
 30. Lee, S.-H., and Tseng, S.C.G. Amniotic membrane transplantation for persistent epithelial defects with ulceration. *Am J Ophthalmol* **123**, 303, 1997.
 31. Ramakrishnan, K.M., and Jayaraman, V. Management of partial-thickness burn wounds by amniotic membrane: a cost-effective treatment in developing countries. *Burns* **23 Suppl 1**, S33, 1997.
 32. Shortt, A.J., Secker, G.A., Lomas, R.J., Wilshaw, S.P., Kearney, J.N., Tuft, S.J., and Daniels, J.T. The effect of amniotic membrane preparation method on its ability to serve as a substrate for the *ex-vivo* expansion of limbal epithelial cells. *Biomaterials* **30**, 1056, 2009.
 33. Hermans, M.H. Preservation methods of allografts and their (lack of) influence on clinical results in partial thickness burns. *Burns* **37**, 873, 2011.
 34. Singh, R., Purohit, S., and Chacharkar, M.P. Effect of high doses of gamma radiation on the functional characteristics of amniotic membrane. *Radiat Phys Chem* **76**, 1026, 2007.
 35. Vangsness, C.T., Jr., Garcia, I.A., Mills, C.R., Kainer, M.A., Roberts, M.R., and Moore, T.M. Allograft transplantation in the knee: tissue regulation, procurement, processing, and sterilization. *Am J Sports Med* **31**, 474, 2003.
 36. Dillow, A.K., Dehghani, F., Hrkach, J.S., Foster, N.R., and Langer, R. Bacterial inactivation by using near- and supercritical carbon dioxide. *Proc Natl Acad Sci U S A* **96**, 10344, 1999.
 37. Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P. Resistance of *Bacillus endospores* to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev* **64**, 548, 2000.
 38. Rosario, D.J., Reilly, G.C., Ali Salah, E., Glover, M., Bullock, A.J., and MacNeil, S. Decellularization and sterilization of porcine urinary bladder matrix for tissue engineering in the lower urinary tract. *Regen Med* **3**, 145, 2008.
 39. Huang, Q., Dawson, R.A., Pegg, D.E., Kearney, J.N., and Macneil, S. Use of peracetic acid to sterilize human donor skin for production of acellular dermal matrices for clinical use. *Wound Repair Regen* **12**, 276, 2004.
 40. Movasaghi, Z., Rehman, S., and Rehman, I.U. Fourier transform infrared (FTIR) spectroscopy of biological tissues. *Appl Spectrosc Rev* **43**, 134, 2008.
 41. Nishida, Y., Yoshida, S., Li, H.J., Higuchi, Y., Takai, N., and Miyakawa, I. FTIR spectroscopic analyses of human placental membranes. *Biopolymers* **62**, 22, 2001.
 42. Ma, D.H., Lai, J.Y., Cheng, H.Y., Tsai, C.C., and Yeh, L.K. Carbodiimide cross-linked amniotic membranes for cultivation of limbal epithelial cells. *Biomaterials* **31**, 6647, 2010.
 43. Meinert, M., Eriksen, G.V., Petersen, A.C., Helmig, R.B., Laurent, C., Uldbjerg, N., and Malmstrom, A. Proteoglycans and hyaluronan in human fetal membranes. *Am J Obstet Gynecol* **184**, 679, 2001.
 44. Burgeson, R.E., El Adli, F.A., Kaitila, II, and Hollister, D.W. Fetal membrane collagens: identification of two new collagen alpha chains. *Proc Natl Acad Sci U S A* **73**, 2579, 1976.

45. Kanayama, N., Terao, T., Kawashima, Y., Horiuchi, K., and Fujimoto, D. Collagen types in normal and prematurely ruptured amniotic membranes. *Am J Obstet Gynecol* **153**, 899, 1985.
46. Malak, T.M., and Bell, S.C. Distribution of fibrillin-containing microfibrils and elastin in human fetal membranes: a novel molecular basis for membrane elasticity. *Am J Obstet Gynecol* **171**, 195, 1994.
47. Higa, K., Shimmura, S., Shimazaki, J., and Tsubota, K. Hyaluronic acid-CD44 interaction mediates the adhesion of lymphocytes by amniotic membrane stroma. *Cornea* **24**, 206, 2005.

Address correspondence to:
Robert J. Christy, PhD
Extremity Trauma Research and Regenerative Medicine
U.S. Army Institute of Surgical Research
JBSA Fort Sam Houston, TX 78234

E-mail: robert.j.christy12.civ@mail.mil

Received: May 27, 2014
Accepted: December 1, 2014
Online Publication Date: March 4, 2015